Evaluation of DNA damage repair gene alterations, microsatellite instability status, and tumor mutational burden as predictive factors of olaparib sensitivity in gastric cancer

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Directed by Professor Sun Young Rha

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ABSTRACT

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One of the many factors that causes cancer is a dysfunction of the DNA damage response pathway (DDR). Cancers with homologous recombination (HR) deficiency due to mutation, methylation, or other reasons, are known to be increased the sensitivity of PARP inhibitors and genomic instability. Besides, DDR, like mismatch repair (MMR), deficiency cancers have been recently reported to have similar features. Furthermore, some studies suggested that many of hypermutated cancers, such as microsatellite instability-high (MSI-H) or high tumor mutational burden (TMB) are DDR deficient. Nevertheless, many studies still have focused on HR-related gene mutations as a predictor of olaparib. Here, we aim to determine whether the DDR gene alterations and genomic instability markers can predict olaparib efficacy in 49 gastric cancer cell lines.

We profiled the genomic status of selected DDR genes, MSI status, and TMB using targeted sequencing. *BRCA1*, *RAD51C,* and *MLH1* methylation were detected by bisulfite sequencing. We separated cell lines as an altered group when it had the truncated mutation, homozygous deletion, or methylation of more than 40% in those genes. RAD51C and MMR-related protein expression was determined by western blot. Then, to determine the efficacy of olaparib, cells were treated with olaparib for 5 days and assessed using CCK-8 assay. Cell

lines were classified as a sensitive group when it had less than 10 μM of IC_{50} and more than 50% of inhibition rate at 10μM.

As a result, twenty of 49 cell lines had the alteration in one or more of the 16 DDR-related genes. In our cell line panel, twelve cell lines were sensitive to olaparib, and DDR without NHEJ altered group was more sensitive to olaparib than the wild type group (p = 0.004). In detail, cell lines with alterations in the HR and MMR subpathways were significantly sensitive among the subtype of DDR pathways respectively, p = 0.005 and p = 0.018. TMB level was widely distributed among the GC cell lines (range = 1.45 to 61.03, median = 8.70), and the DDR altered without NHEJ group had higher TMB than wild type group (p = 0.021). Four cell lines (SNU-1, SNU-638, IM95m, and NUGC-3) were MSI-H, and all of them were no MLH1 protein expression. MSI-H cell lines had significantly higher TMB than other cell lines (median : 47.96 vs 8.72, p < 0.0001). Three MSI-H cell lines were sensitive to olaparib, but NUGC-3 with the lowest TMB (29.07mt/mb) among MSI-H cell lines was resistant. As a result of analyzing the importance and olaparib sensitivity predictive ability of each factor, core HR alteration and DDR alteration excluding NHEJ were similarly important (2.53 vs 2.48), and the area under the curve (AUC) was the largest for DDR alteration excluding NHEJ.

In our result, the olaparib sensitive group had the alteration in DDR genes and high TMB. MSI-H cell lines were sensitive to olaparib when it had high TMB. When we analyzed single factors and combined scores, the predictive ability of olaparib sensitivity was better with DDR alteration excluding NHEJ. For accurate predictions that are sensitive to olaparib, our data suggest that it is necessary to analysis DDR alteration by extending core HR alteration.

Key words : PARP inhibitor, homologous recombination deficiency, DNA damage repair deficiency, genomic instability marker, gastric cancer

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I. INTRODUCTION

According to the National Cancer Center from Korea, diagnosed cancer and deaths from cancer were reported as 243,837 and 79,153 during 2018 and show an increasing trend every year.[1] Gastric cancer (GC) has the highest incidence (11.45%), and conventional chemotherapy has shown limited efficacy with a median survival of 10 months.[2]

One of the characteristics of cancer is the accumulation of mutations resulting from the dysfunction of the DNA repair system. Damaged DNA by endogenous and exogenous factors is repaired through the DNA repair pathway that many proteins are involved in. If mutations in these proteins occur, the function of the DNA repair pathway is declined and the risk of cancer is increased. Dysfunction of the DNA repair pathway is one of the hallmarks of cancer, and DNA damage accumulate in the cancer genome.[3]

Damaged DNA by endogenous and exogenous sources in normal cells is recovered via repair pathways. DNA damage response (DDR) system is largely classified as non-homologous end joining (NHEJ) and homologous recombination (HR) to repair double-strand breaks (DSB), and base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) to repair single-strand breaks (SSB).[4] Among the many DNA repair enzymes, PARP is an

enzyme that recognizes SSB and induces BER. PARP-1 and PARP-2 are known to play an important role in the DNA repair pathway.[5,6]

PARP inhibitor leads to trapping of PARP proteins which recognize SSB site, and induce synthetic lethality in HR deficient cancer such as those caused by *BRCA1/2* mutations.[7] SSB breaks not repaired by PARP inhibitor are unstable and it is lead to DSB, which could be repaired through an HR pathway that does not cause DNA sequencing errors based on the sister chromatin.[8] However, synthetic lethality causes unlike normal cells, when PARP inhibitor is treated on cancer with mutations in genes that play an important role in the HR pathway.[9]

Olaparib was the first FDA-approved PARP inhibitor for ovarian cancer patients with germline mutations in *BRCA1* and *BRCA2*, and later, FDA approval for a variety of carcinomas, including metastatic castration-resistant prostate cancer patients.[10] However, besides *BRCA1* and *BRCA2*, many related factors affect the homologous recombination pathway. In addition to genetic mutation, functional degradation due to various factors such as methylation has also been reported.[11] For this reason, PARP inhibitors are expected to be effective in cancer patients with not only mutations in *BRCA1* and *BRCA2* germline mutations, but also mutations and methylation of various related genes. Related research is also being actively conducted, but research on gastric cancer is still insufficient.

In addition, genomic instability caused by damage to the DNA repair pathway has been studied variously as a major feature of cancer. Typically, genomic instability can be explained by the change in the length of microsatellite (MSI status) and the tumor mutational burden (TMB), which refers to the number of sporadic somatic mutations throughout the genome.[12,13] Although it is known to be high in cancers with mutations, the association with PARP inhibitor sensitivity has not yet been revealed in gastric cancer.

Therefore, this study comprehensively analyzes genomic, epigenetic, and molecular profiling of DDR related genes and genomic instability markers using a GC cell line panel to confirm the association with olaparib sensitivity.

II. MATERIALS AND METHODS

1. Materials

Olaparib was purchased from Selleckchem (TX, USA). The primary antibody for western blot against MLH1 (EPR3894) was purchased from Abcam (Cambridge, UK), while MSH2 (G219-1129) and MSH6 (44) were purchased from BD Biosciences (MA, USA). RAD51C (2H11/6) antibody was purchased from Novus Biologicals (CO, USA). HRP-conjugated anti-mouse and anti-rabbit were used for the secondary antibody. Anti-α-tubulin antibody (Sigma Aldrich, MO, USA) was used for normalization.

The primary antibody for immunofluorescence against RAD51 (F-11) was purchased from Santa Cruz Biotech (CA, USA). Goat anti-mouse Alexa fluor 488 and goat anti-rabbit Alexa fluor 594 which fluorescent-dye conjugated secondary antibodies (PA, USA) were used for labelling of primary antibody.

2. Cell lines and culture

Four human gastric cancer cell lines were purchased from American Type Culture Collection (ATCC, MD, USA), eleven cell lines were purchased from Korean Cell Line Bank (KCLB, Seoul, South Korea), nine cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan), and twenty-five cell lines were established by Songdang Institute for Cancer Research (SICR, Seoul, South Korea) from metastatic gastric cancer patients who visited Yonsei Cancer Center. Two human breast cancer cell lines, HCC-1937 and MCF-7, and a colon cancer cell line LoVo, were purchased from the ATCC. Cells were cultured in Minimum Essential Medium (EMEM),

Roswell Park Memorial Institute· 1640 (RPMI· 1640) medium, or Dulbecco Modified Eagle's Medium (DMEM) (MA, USA) containing 10% fetal bovine serum (Lonza, Basel, Switzerland), 100 units/mL of penicillin and 100μg/mL of streptomycin (Lonza) at 37℃ in 5% CO_2 incubator.[14]

3. In-house deep sequencing and RNA sequencing data analysis

Genomic DNA of cell lines was extracted using a Blood Genomic DNA Isolation Kit Mini (COSMO genetech, Seoul, South Korea). In-house deep sequencing was performed at Celemics (Seoul, South Korea) to use CancerMaster Panel V2.[15] RNA sequencing data of the 49 GC cell lines were obtained from the genome database of SICR. The mRNA expression levels were measured in fragments per kilobase (kb) of exon model per million mapped reads (FPKM) without normalization.

4. Bisulfite modification and pyrosequencing

EZ DNA methylation kit (ZYMO Research, CA, USA) was used for bisulfite conversion of genomic DNA according to the manufacturer's instructions. Bisulfite-modified DNA was carried on one-step PCR for *BRCA1* and *RAD51C*, and two-step PCR for MLH1. Primer design was based on the PyroMark Q24 software (QIAGEN, Hilden, Germany). Primer sequences are shown in Table 1.[16,17] *BRCA1*, *RAD51C*, and *MLH1* methylation were determined by pyrosequencing (PyroMark Q24, QIAGEN). The methylation percentage was defined as the average of each CpG island methylation percentage. If the cell lines methylated more than 10%, it was classified as having methylation.

Table 1. The sequences of the bisulfite sequencing primers for PCR and pyrosequencing.

Gene	Primer sequence (5' → 3')	Length (bp)	AT (℃)
BRCA1	F : GGT AGA TTG GGT GGT TAA TTT AGA R : CTA AAA AAC CCC ACA ACC TAT CC Seq : GAA TTA TAG ATA AAT TAA AA	232	60
RAD51C	F : TTG GTT GTT TYG GGG TTA GTA GGT R : CAC CTC TAA AAA TTC CTC AAC AAT CTA AA Seq : GGG GTT AGT AGG TGA	156	60
MLH1	1st F : TAG TAG TCG TTT TAG GGA GGG A 1st R : TCT AAA TAC TCA ACC AAA ATA CCT T	194	50
	2nd F : TTG GTA TTT AAG TTG TTT AAT TAA TAG TTG 2nd R : GGG ACA CCG CTG ATC GTT TAA AAA TAC CTT CAA CCA ATC ACC TC Universal : GGG ACA CCG CTG ATC GTT TA Seq : AGT TAT AGT TGA AGG AAG AA	119	45

5. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA extraction was extracted using Trizol reagents (Invitrogen, CA, USA), and cDNA synthesis was performed on 500ng of total RNA with SuperScript™ II Reverse Transcriptase (Invitrogen, CA, USA). Real-time PCR was performed using QuantiTect SYBR® Green PCR Kits (Qiagen, Germany) as follows: 40 cycles of 30 seconds at 95 ℃, 30 seconds at 60 ℃ (for RAD51C) and 57℃ (for GAPDH), and 30 seconds at 60 ℃. The sequence of primers was as follows: RAD51C-forward, 5' CCT CCG AGC TTA GCA AAG AA 3'; reverse, 5' CCA CCC CCA AGA ATA TCA TC 3'.[18] Delta-delta Ct method after normalization with GAPDH was used to calculate the mRNA expression. RAD51C mRNA expression under normal condition was quantified based on RAD15C unmethylated control, SNU-668. The mRNA fold change was calculated by dividing the mRNA expression in olaparib treatment by the mRNA expression in the normal condition.

6. Cell viability assay

Cells were seeded at a density of 1 to 4 $\times$ 10^3 in 96-well plates. After 24 hrs of incubation, the cells were treated with DMSO which was a vehicle or olaparib for 5 days. The Cell Counting Kit- 8 solution (CCK- 8; Dojindo, Kumamoto, Japan) was added to each well and the plates were incubated for 2 hrs. Cell viability measured the absorbance at 450nm using a Microplate reader (Tecan, Switzerland).[18] IC_{50} values were calculated with CalcuSyn software (Biosoft, Cambridage, UK).

7. Western blot analysis

Cells were lysed in the M-PER mammalian protein extraction reagent (Pierce, IL, USA) containing phosphatase inhibitor (Sigma, MO, USA) and protease inhibitor (Roche, Basel, Switzerland). Protein concentrations were determined by the Bradford Assay (Bio-Rad, Hemel Hempstead, UK). Proteins were separated by 8-15% SDS-PAGE and transferred to PVDF membranes. Non-specific antibody binding was reduced by blocking process with 5% skim milk at 2 hrs incubation. Membranes were incubated overnight at 4℃ with primary antibodies. After 6 times of wash, membranes were incubated for 1 hr for room temperature with HRP-conjugated secondary antibody. Immunoblots were developed using a LumiFlashTM Ultima Chemiluminescent Substrate (Visual Protein, Neihu Dist, Taiwan) and visualized by ChemiDoc XRS+ System (Bio-Rad).

8. Immunofluorescence

Cells were seeded on 8-well cell culture slide (SPL Life Sciences, Korea) for 24 hours, followed by exposure to 5μM olaparib for 24 hours. Then, cells were fixed with 4% formaldehyde for 10 minutes, permeablized with 0.1% Triton X-100 for 10 minutes, and blocked by 0.2% BSA for 1 hour. After blocking, cells were incubated overnight at 4℃ with primary antibodies and were incubated for 1 hour at room temperature with secondary antibodies. Finally, cells were stained using 300nM DAPI. The slides were observed under an LSM700 (ZEISS, Germany). Images were taken over 5 points randomly, and nuclei and RAD51 foci were analyzed with Zen lite (blue edition) software 3.0 (ZEISS). When DAPI-stained nucleic acids had 6 or more RAD51 foci, they were classified as RAD51 foci positive cell lines. RAD51 foci

change was calculated by dividing the ratio of RAD51 foci positive cell lines after olaparib treatment by the ratio of RAD51 foci positive cell lines before olaparib treatment.

9. Statistical analysis

Statistical analyses were performed using SPSS software version 25.0 (SPSS Inc., IL, USA). Comparison between groups was analyzed by the unpaired Student t-tests and one-way ANOVA when variables were normally distributed, while Mann-Whitney U test and Kruskal-Wallis test were performed when variables were not normally distributed. Logistic regression was used to analyze independent predictors. The beta coefficients from the logistic regression model were used to build point-based combined scores. The score was assigned to each predictor by multiplying the regression coefficient. The weighted combined score was obtained from the sum of the score of each predictor and rounding it to the nearest integer. A receiver operating characteristic curve (ROC) analysis was according to the sensitivity and specificity of each factor. Statistical significances were determined by less than 0.05 the p-value.

III. RESULTS

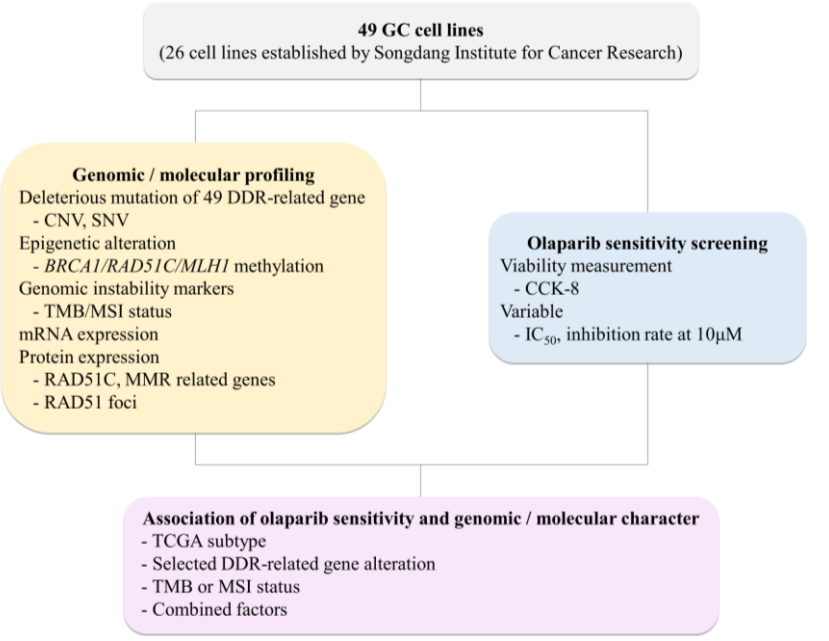


Figure 1. Study scheme. Forty-nine GC cell lines were used in this study.

The study was designed to determine whether DDR gene alteration and genomic instability markers could predict olaparib efficacy in GC. We confirmed the mutations of the selected 49 DDR-related genes and the methylation status of *BRCA1*, *RAD51C* and *MLH1*. In addition, TMB and MSI status were defined as genomic instability markers. The association between each factor and olaparib sensitivity was analyzed through genomic and molecular profiling results and olaparib sensitivity screening results.

HR (and FA) (n=33)			MMR (n=5)	BER/NER (n=12)	NHEJ (n=3)
ATM	*CHEK1*	*MRE11*	*MLH1*	*APEX1*	*ARID1A*
ATR	*CHEK2*	*PALB2*	*MSH2*	*ATR*	*PRKDC*
ATRX	*FANCA*	*RAD50*	*MSH6*	*CUL4A*	*RAD50*
BACH1	*FANCC*	*RAD51*	*PMS2*	*MUTYH*	
BAP1	*FANCD2*	*RAD51B*	*POLD1*	*PARP1*	
BARD1	*FANCE*	*RAD51C*		*PARP2*	
BLM	*FANCF*	*RAD51D*		*PARP3*	
BRCA1	*FANCG*	*RAD52*		*PARP4*	
BRCA2	*FANCI*	*RAD54L*		*POLD1*	
BRIP1	*FANCL*	*RPA1*		*POLE*	
CDK12	*FANCM*	*XRCC3*		*RPA1*	
				TIPARP	

Table 2. Selected DDR genes. Genes were selected as those reposted to be involved in DNA damage repair or to affect PARP inhibitor sensitivity.

Gene	AA change*	Cell lines
ATM	V1153fs	SNU-1
BRCA2	K1530fs	SNU-1
	V3082fs	SNU-638
BRCA1	Q541X	SNU-668
BARD1	K208fs	IM95m
	E580X	SNU-16
RAD51B	Q28X	YCC-3/7
RAD54L	R552X	YCCEL1/YCC-10
BAP1	HOMDEL	NUGC-4
FANCA	S103X	YCC-27
XRCC3	H322fs	IM95m
RPA1	G178fs	NUGC-3
ATR	I774fs	IM95m, SNU-638
APEX1	R181X	SNU-638
MLH1	R226X	SNU-1
MSH6	T1355fs	SNU-16, SNU-5
	T1085fs	SNU-638
ARID1A	G1847fs	IM95m, NUGC-3, SNU-1
	I1130fs	OCUM-1
	A1517fs	SNU-1
	G370fs	SNU-1
	Q1458X	SNU-216
	G921fs	SNU-638
	R693X	YCC-17
	E1964X	YCC-23
	K2124X	YCC-38
	HOMDEL	SNU-5, YCC-6

Table 3. Deleterious mutations in GC panel. * : amino acid change. The genes

were arranged in order of importance.

We selected 49 DDR genes that were be involved either in DNA damage repair or PARP inhibitors sensitivity.[12,19-22] (Table 2) DDR genes were classified into HR including FA family, MMR, BER and NER (BER/NER), and NHEJ according to the DDR subpathways. Among the HR genes, *BRCA1*, *BRCA2*, *ATM* and *RAD51C* were defined as core HR.[18,19] For the landscape of selected DDR mutations in 49 GC cell lines, we used an in-house deep sequencing panel (524 genes). We identified it as a deleterious mutation if cell lines had truncated mutation, homozygous deletion, and missense mutation which was known to cause loss of function. Fifteen of forty-nine cell lines had a deleterious mutation in one or more DDR-related genes. Of these, 13 genes had only deleterious mutation type, only BAP1 had homozygous deletion, and ARID1A had both types. Interestingly, nonsynonymous mutations were the most common mutation type out of all mutations, of which none were known to caused loss of function. The most frequently altered gene was *ARID1A* (22.4%, 11/49). (Table 3)

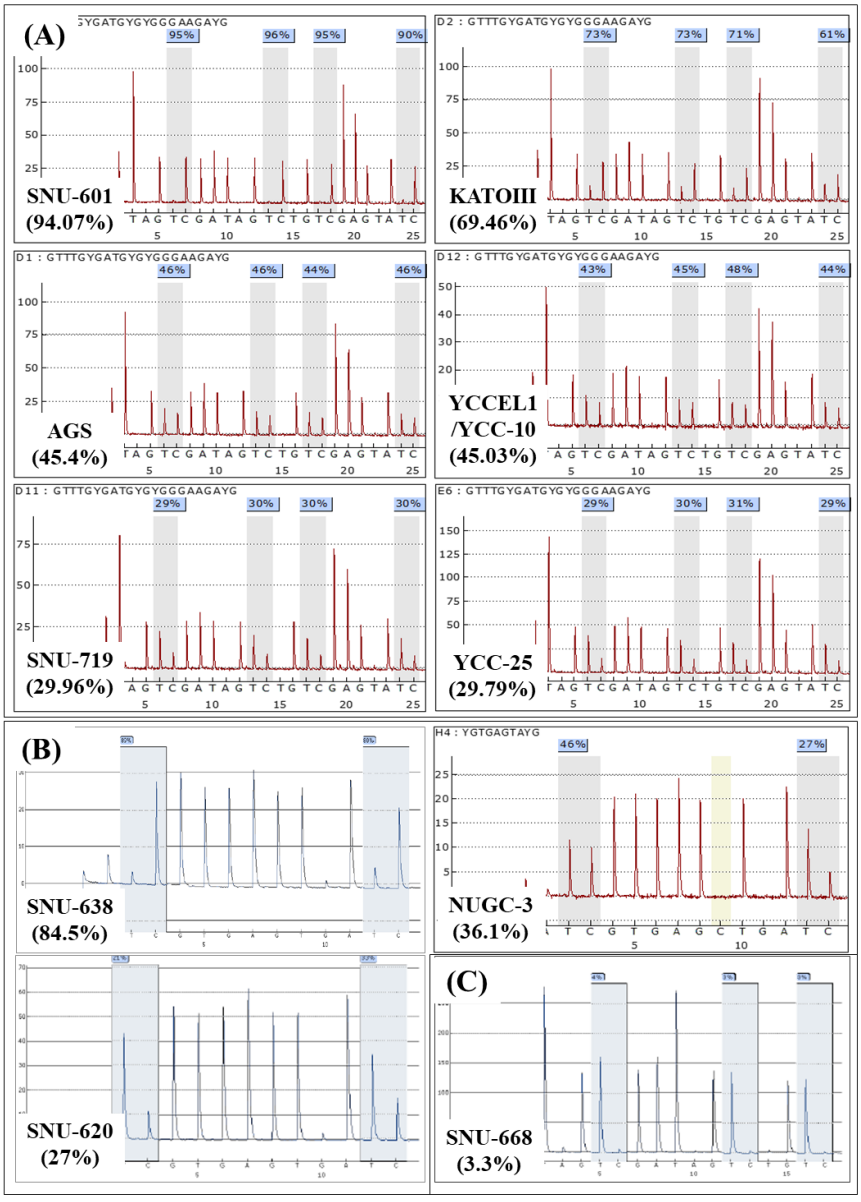


Figure 2. Measurement of the *RAD51C*, *MLH1*, and *BRCA1* methylation using pyrosequencing. (A) Result of *RAD51C* methylation status. Six cell lines were methylated more than 10%. (B) Result of *MLH1* methylation status. Three cell lines were methylated more than 10%. (C) Result of *BRCA1* methylation status. There were no *BRCA1* methylated cell lines in 49 GC cell lines.

Next, we determined the levels of *BRCA1*, *RAD51C*, and *MLH1* methylation. In 49 GC cell lines, *BRCA1* was not methylated, but *RAD51C* was methylated by more than 10% in 6 cell lines (SNU-601, KATOIII, AGS, YCCEL1/YCC-10, SNU-719, and YCC-25), and *MLH1* was methylated by more than 10% in 3 cell lines (SNU-638, NUGC-3, and SNU-620) (Fig 2). The average methylation percentage of methylated cell lines with *RAD51C* was 48.95%, and *MLH1* was 49.37%, while unmethylated cell lines with *RAD51C* was 0.98% and *MLH1* was 2.7%.

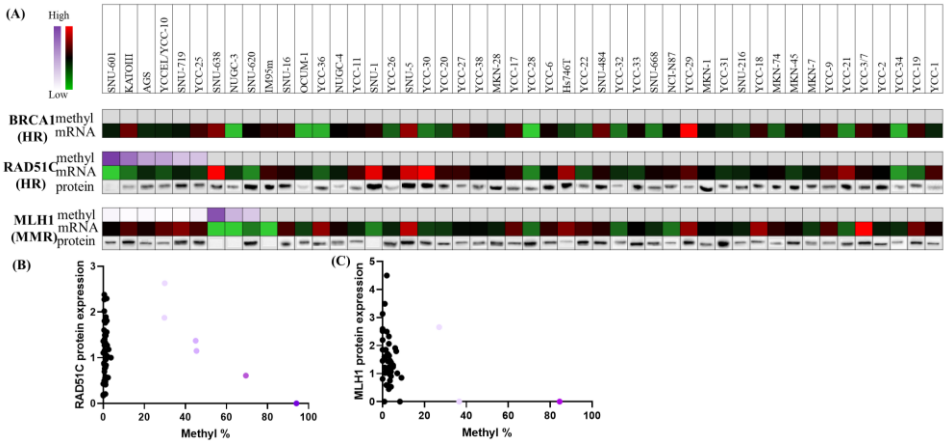
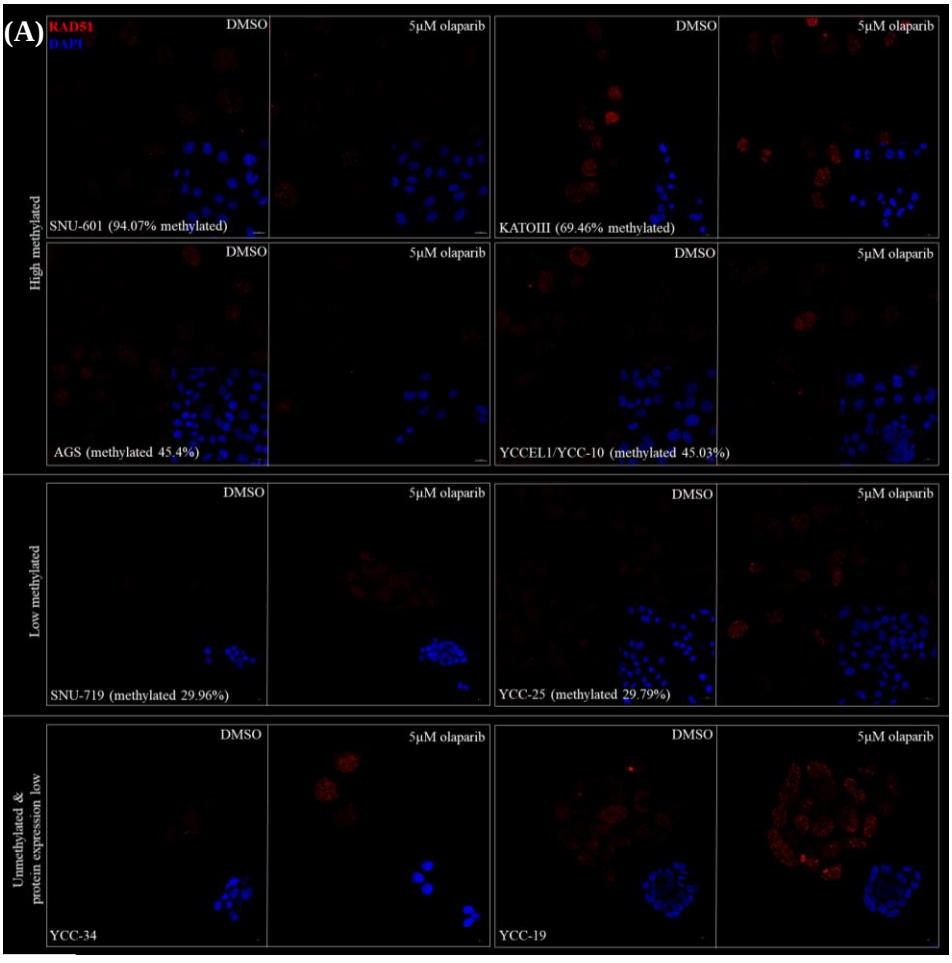


Figure 3. mRNA expression using RNA sequencing, and protein expression using western blot. (A) *BRCA1*, *RAD51C*, and *MLH1* methylation status, mRNA expression, and protein expression in 49 GC cell lines. (B) The correlation between RAD51C protein expression and methylation percentage. (C) The correlation between MLH1 protein expression and methylation percentage.

mRNA and protein expression is regulated for several mechanisms, such as methylation. Therefore, mRNA and protein expression were checked to confirm the expression difference according to the methylation level. As a result of comparative analysis of mRNA and protein expression with methylation status, protein expression was lower than the average when methylation was 40% or more.



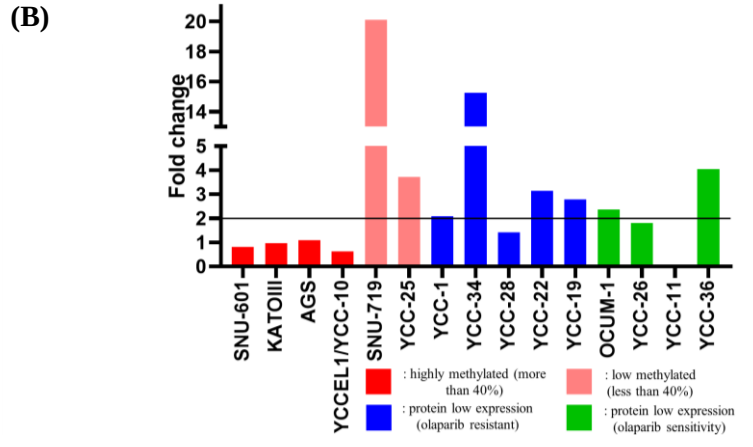


Figure 4. RAD51 foci which were an HR function marker in RAD51C methylated and RAD51 protein low expression cell lines. Cells were exposed at 5μM of olaparib for 24 hrs. (A) RAD51 foci. (B) The fold change of RAD51 foci changes after olaparib expose.

To define a significant alteration, we selected cells with RAD51C methylation (SNU-601, KATOIII, AGS, YCCEL1/YCC-10, SNU-719 and YCC-25) and cells with low RAD51C protein expression. Cell lines with low protein expression were further divided into olaparib resistant (YCC-1, YCC-34, YCC-28, YCC-22 and YCC-19) and sensitive group (OCUM-1, YCC-26, YCC-11 and YCC-36). Unmethylation cell lines were all selected as cell lines with lower protein expression than control and lower mRNA expression under similar conditions. RAD51 foci, a representative HR function marker, were generated at the DSB site. RAD51 foci were identified by immunofluorescence to determine the effect of methylation percentage or protein expression on the HR function. Methylated more than 40% cell lines cell lines showed no change in RAD51 foci formation upon olaparib treatment, but methylated less than 40% cell lines increased RAD51 foci formation. Similarly, in the case of low protein expression and olaparib resistant cell lines, RAD51 foci formation was increased upon olaparib treatment, excepted YCC-28 which had high RAD51 foci positive rate in DMSO treatment. Protein expression low and olaparib sensitive cell lines showed different characteristics.

Since it was confirmed that HR function was weakened according to the degree of methylation regardless of protein expression, if cell lines were methylated more than 40%, it was included as an altered group.

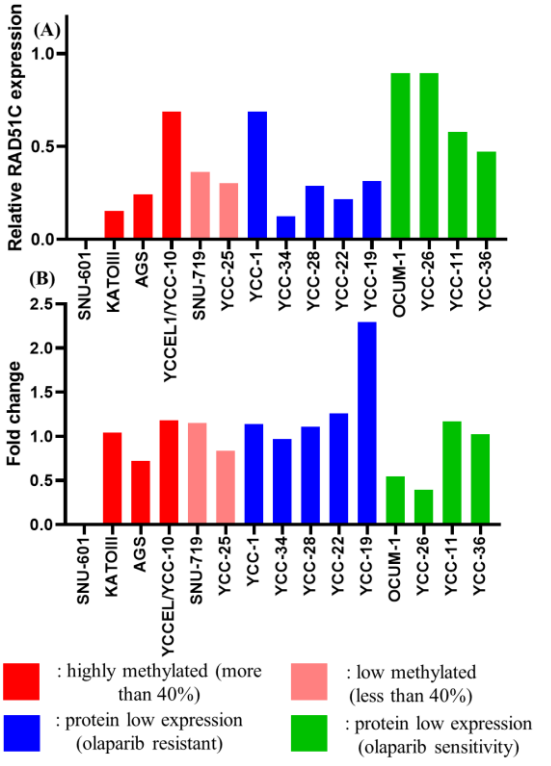


Figure 5. RAD51C mRNA expression in each subgroup. (A) mRNA expression of each cell line. (B) The fold change of mRNA expression changes after olaparib expose. Cells were exposed at 5μM of olaparib for 72 hrs.

We hypothesized that the change in RAD51C expression in cell lines with low RAD51C protein expression during olaparib treatment was the cause of the difference in HR function between cell lines with low protein expression and cell lines with high methylation. First, we observed mRNA expression in the normal condition. The comparison was made based on SNU-668, a RAD51C unmethylated control. Methylation control, SNU-601, did not have RAD51C mRNA expression and there was no difference in each subgroup. After 72 hours of treatment with Olaparib, there was no difference in RAD51C mRNA expression for each subgroup.

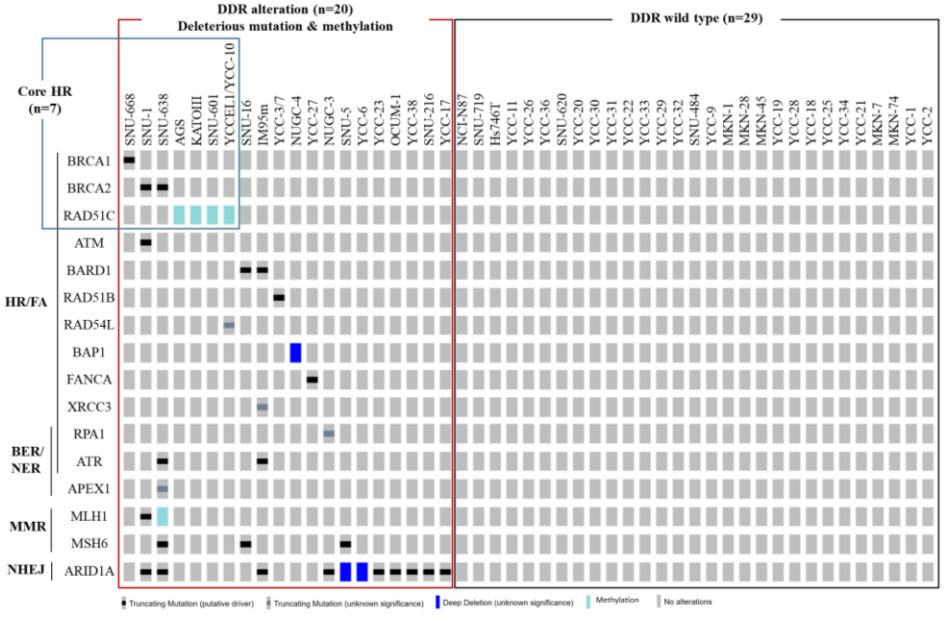


Figure 6. DDR related gene alteration in 49 GC cell lines. If the cell lines had a deleterious mutation or more than 40% of methylation, it was classified as DDR altered group.

Alteration in this study was defined as a deleterious mutation in 49 DDR related genes, and more than 41% methylation in *BRCA1, RAD51C* and *MLH1*. Of the 49 GC cell lines, 20 cell lines were classified as altered groups since they had one or more alteration. The most frequently altered gene was *ARID1A* (22.4%, 11/49), followed by *RAD51C* (8.2%, 8/49) in the 49 GC. Interestingly, in the case of RAD51C, there was no deleterious mutation, only methylation. Of the 20 DDR altered groups, 13 cell lines had alterations in HR. There were 4 MMR altered cell lines, and 2 of them also had an alteration in HR. All three cell lines in the BER/NER altered group also had alterations in HR. In contrast, only 5 of 11 cell lines in the NHEJ alt group had an alteration in HR.

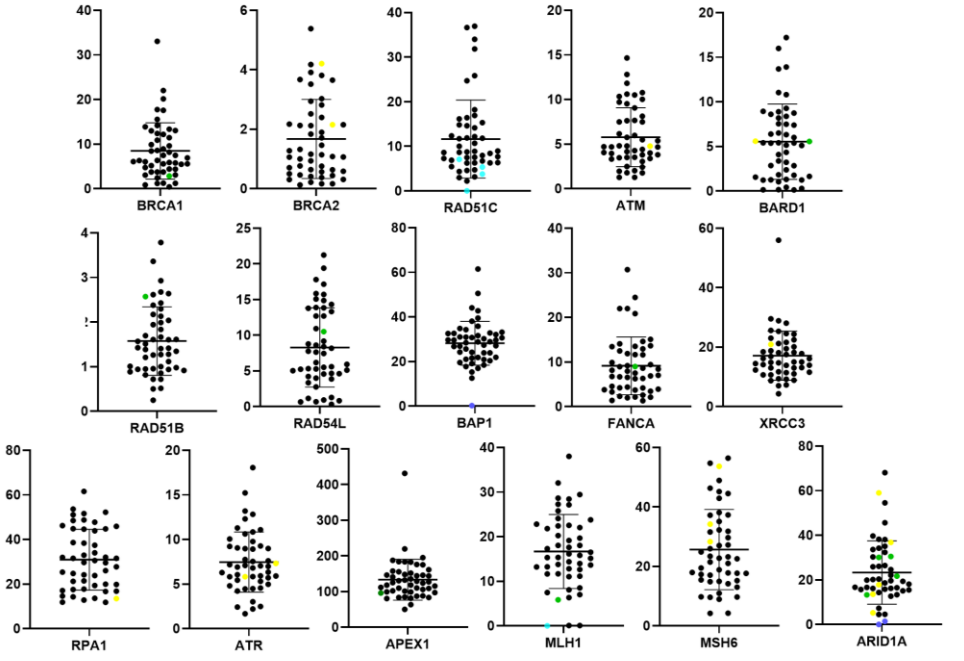


Figure 7. mRNA expression of the gene carrying alteration. The Y-axis value was FPKM. Yellow : stop gain, green : frameshift mutation, dark blue : homozygous deletion, light blue : methylation

mRNA expression according to the alteration type was confirmed. Among alteration types, stop gain and frameshift, which are truncated mutations, did not significantly affect mRNA expression even with an alteration. However, in the case of methylation (in *RAD51C* and *MLH1*), mRNA expression was generally low, and in the case of homozygous deletion (in *BAP1* and *ARID1A*), there was no mRNA expression.

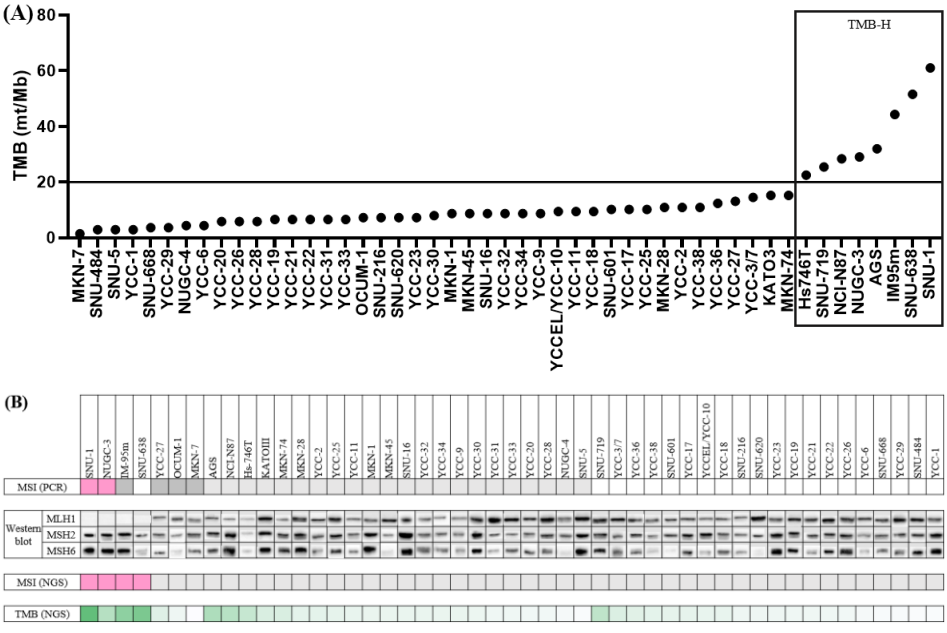


Figure 8. Distribution of TMB based on targeted sequencing data and MSI status to use several methods in 49 GC cell lines. (A), (E) Distribution of TMB. The cell line which had higher than 10 was defined as TMB-H. (B) Result of MSI status based on 5-marker PCR to use extracted DNA from tumor tissue. (C) MMR related protein expression by western blot. (D) MSI status based on targeted sequencing data.

TMB is a marked for genomic instability that was counted the total number of somatic mutations by sequencing. It was widely distributed among the GC cell lines (range = 1.45 to 61.03), and the median TMB was 8.70. TMB-high (TMB-H) was defined if the TMB of the cell line was 20 or more, and TMB-low (TMB-L) was defined if the TMB was below 20 in the CancerMaster panel. According to the standard, 8 cell lines (16.3%) were classified as TMB-H (median = 20.518). (Fig 8A)

Since MSI status was also a representative genomic instability marker, it was analyzed by several methods. First, we performed 5-marker PCR to use paired

tumor and normal tissues DNA which was the traditional MSI status detection method. The cell lines were classified into three categories, MSI-H was defined if two or more unstable markers were detected, MSI-L was defined if only one unstable marker was detected, and MSS was defined if cell lines didn't have any unstable marker. Of the 49 GC cell lines, twenty-six cell lines had MSI status results judged at the clinical level. (Fig 8B) Moreover, protein expression of MLH1, MSH2, and MSH6 were MMR related proteins by western blot. Four cell lines (SNU-1, SNU-638, IM95m, and NUGC-3) were no MLH1 protein expression, while MSH2 and MSH6 were expressed in all of the cell lines. (Fig 8C) Finally, when analyzed using targeted sequencing data, four cell lines were classified as MSI-H. (Fig 8D) In the case of IM95m which defined MSI-H to use targeted sequencing data, it was clinically defined as MSI-L, but since one of the five markers was failed to detect, the possibility of MSI-H could not be excluded, and the MLH1 protein expression was also lost. Besides, in the case of SNU-638, there was no clinical test result, but it was classified as MSI-H based on targeted sequencing, and the MLH1 protein expression was lost too. So, results of three different methods were synthesized, four cell lines (SNU-1, SNU-638, IM95m, and NUGC-3) were classified as MSI-H in this study. MSI-H cell lines had significantly higher TMB than other cell lines (median : 47.96 vs 8.72, $p < 0.0001$) (Fig 8E).

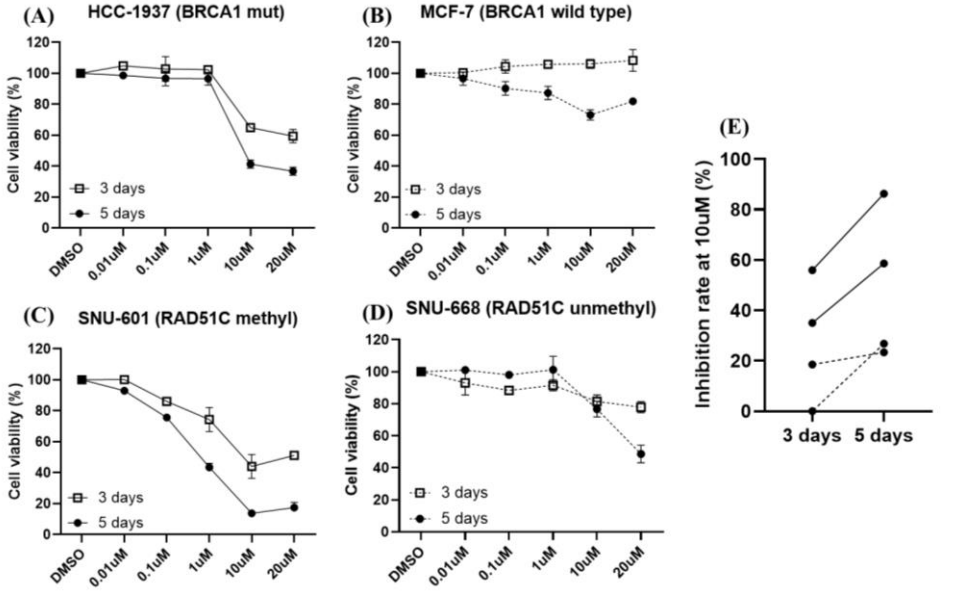


Figure 9. Olaparib sensitivity of control cell lines. (A) Olaparib sensitive breast cancer cell line. HCC-1937 had *BRCA1* (5382insC) (B) Olaparib resistant breast cancer cell lines. MCF-7 was BRCA wild type. (C) Olaparib sensitive gastric cancer cell lines. SNU-601 was methylated in *RAD51C*. (D) Olaparib resistant gastric cancer cell line. SNU-668 was unmethylated in *RAD51C*. (E) Comparison of the inhibition rate at 10μM. Solid line : olaparib sensitive control cell line. Dotted line : olaparib resistant control cell line.

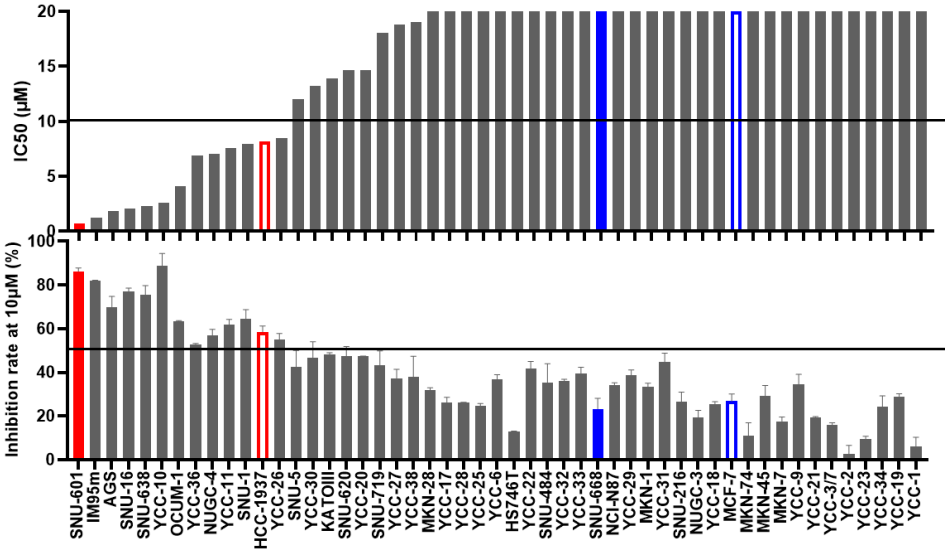
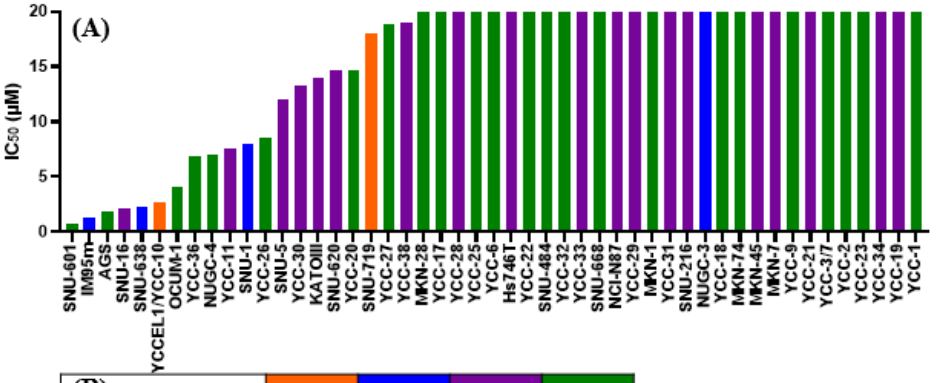


Figure 10. Olaparib sensitivity of 49 GC and 2 breast cancer cell lines. If cell lines had less than 10μM of IC50 and higher than 50% of inhibition rate at 10μM, it classified as olaparib sensitive group.

To assess the efficacy of olaparib on gastric cancer cells, 49 GC cell line panel was exposed to different concentrations of olaparib. The anti-tumor effect was analyzed using a cell growth inhibition assay. Before olaparib sensitivity screening, we tested two breast cancer cell lines, HCC-1937 which had a deleterious mutation in *BRCA1* and MCF-7 which was BRCA wild cell lines, and two gastric cancer cell lines, SNU-601 which methylated in *RAD51C* and SNU-668 which unmethylated in *RAD51C*.[18,23] When cell lines were exposed to olaparib for 3 days, all of the cell lines except SNU-601 was measured IC_{50} value to be more than 20μM which was the screening maximum dose. However, when cell lines were exposed for 5 days, both of olaparib sensitive control cell lines (HCC-1937 and SNU-601) had IC_{50} 8.15μM and 0.74μM each. Otherwise, olaparib resistant control cell lines (MCF-7 and SNU-668) had IC_{50} not less than 20μM. (Fig 9A - 9D) In addition, olaparib sensitive control cell lines showed a higher inhibition

rate when exposed for 5 days than when exposed for 3 days, but resistant cell lines did not increase much (27% vs 15.82%) (Fig 10E).

As a result, twelve cell lines were highly sensitive to olaparib compared with other cell lines (median of inhibition rate at 10μM : 67.3% vs 31.9%). Olaparib sensitive cell lines had IC_{50} less than 10μM and an inhibition rate higher than 50%. IC_{50} of 29 cell lines was not measured. (Fig 10)



(B)	EBV	MSI	GS like	CIN like
Mean IR (%)	66.0	60.4	36.0	37.1
Total cell lines #	2	4	24	19
IR < 50%	1	1	18	17
IR ≥ 50%	1	3	6	2
% sensitive cell lines	50%	75%	25%	10.5%

Figure 11. The comparison of olaparib sensitivity according to TCGA subtype. (A) Olaparib sensitivity and TCGA type of 49 GC cell lines. (B) Average IR and distribution of sensitive cell lines for each TCGA group.

Fifty percentage of the EBV type and 75% of the MSI-H type were sensitive to olaparib (46.03% and 46.45% inhibition rate at 10μM average values, respectively). On the contrary, GS like type and CIN like type were sensitive only 25%, and 10.5% each. (39.62% and 39.13% inhibition rate at 10μM average values, respectively)

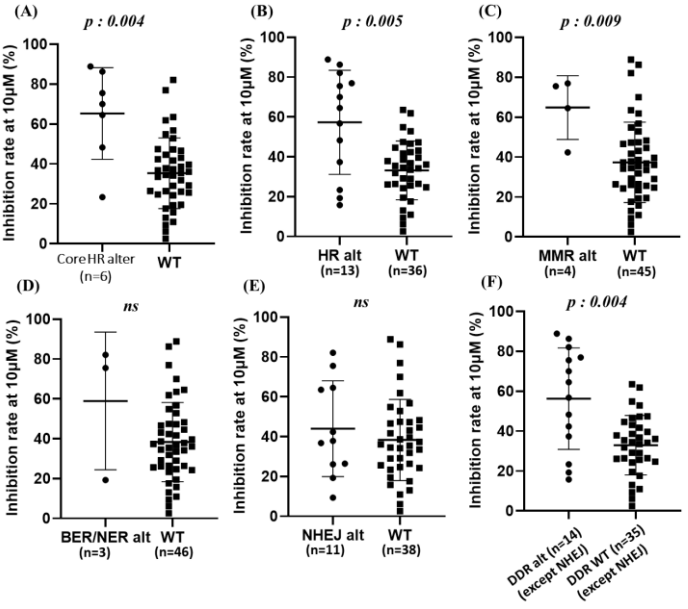


Figure 12. Comparison of olaparib sensitivity according to DDR altered groups. (A) Comparison core HR altered, (B) HR altered, (C) MMR altered, (D) BER and NER altered, (E) NHEJ altered, and (F) DDR altered except for NHEJ and wild type group.

DDR alteration group was more sensitive to olaparib than the DDR wild type group (p = 0.030). In detail, cell lines with alterations in the HR and MMR were significantly sensitive among the subtype of DDR pathways (p = 0.005, p = 0.011). The BER/NER altered group was difficult to analyze due to the number of cell lines with alteration was too small. Nevertheless, eleven cell lines which were a relatively sufficient number to analyze were in NHEJ altered group, the NHEJ altered group did not show a significant difference in olaparib sensitivity from the wild type group. Except for NHEJ, other DDR altered groups were significantly more sensitive when compared to wild type groups (p = 0.004) Three MSI-H cell lines were sensitive to olaparib, but NUGC-3 with the lowest TMB (29.07) among MSI-H cell lines was resistant.

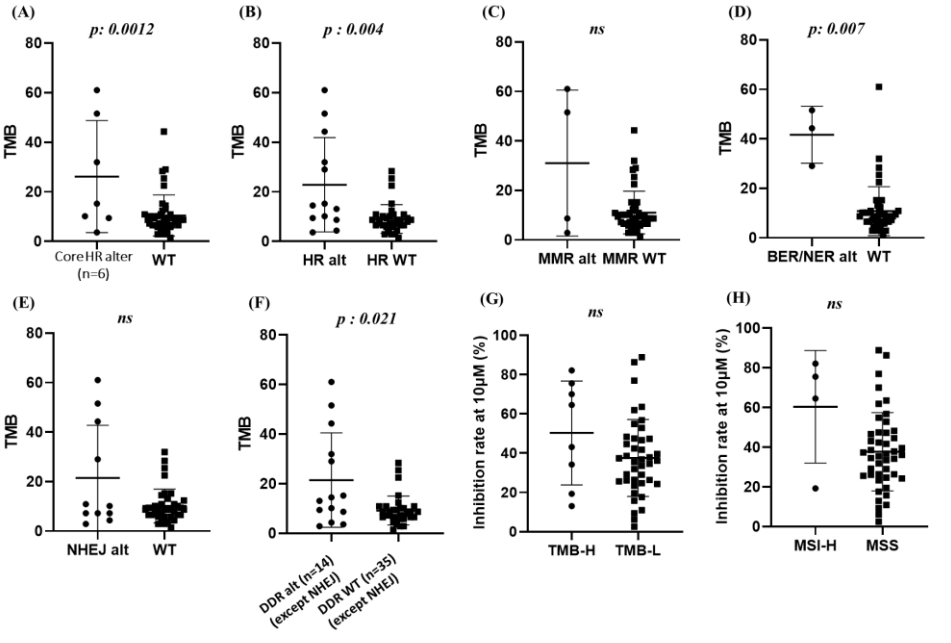


Figure 13. Comparison of TMB according to DDR altered groups, and examination of olaparib sensitivity according to genomic instability markers. (A) Comparison core HR altered, (B) HR altered, (C) MMR altered, (D) BER and NER altered, (E) NHEJ altered, and (F) DDR altered except for NHEJ and wild type group. (G) Comparison of olaparib sensitivity TMB-H and TMB-L group, and (H) MSI-H and MSS group.

Similarly, the NHEJ altered group showed no significant difference compared to the wild type group. Contrary to expectations, there was no significant difference between the MMR altered group and the wild type group. However, when comparing the DDR altered group with the wild type group, except for NHEJ, TMB was significantly higher in the altered group than in the wild type group (p = 0.021). (Fig 13) There was no significant difference in olaparib sensitivity between TMB-H and TMB-L group, as well as MSI-H and MSS group. (Fig 13G, 13H)

Variable	Estimate (β)	OR (95% CI)	*p-value*
Core HR	2.5257	12.500 (2.006 – 77.895)	0.0068
DDR (without NHEJ)	2.3353	10.332 (2.341 – 45.607)	0.0021
MSI-H	2.4849	12.000 (1.113 – 129.416)	0.0406
TMB	1.4171	4.125 (0.844 – 20.159)	0.0800

Table 4. The importance and olaparib sensitivity predictive ability of each factor. Comparison of the importance according to each factor.

Table 5. Overall comparison of each method

		TMB	Cut-off (≥)	Sensitivity	Specificity	PPV	NPV	Accuracy
Single factor	Core HR			41.7%	94.6%	71.4%	83.3%	81.6%
	TMB			33.3%	89.2%	50.0%	80.5%	75.5%
	MSI-H			25.0%	97.3%	75.0%	80.0%	79.6%
	DDR (w/o NHEJ) Binary			66.7%	83.8%	57.1%	88.6%	79.6%
Combined Score 1	DDR(w/o NHEJ) Binary	O	2	66.7%	83.8%	57.1%	88.6%	79.6%
Combined Score 2	DDR(w/o NHEJ) Binary	X	2	66.7%	83.8%	57.1%	88.6%	79.6%

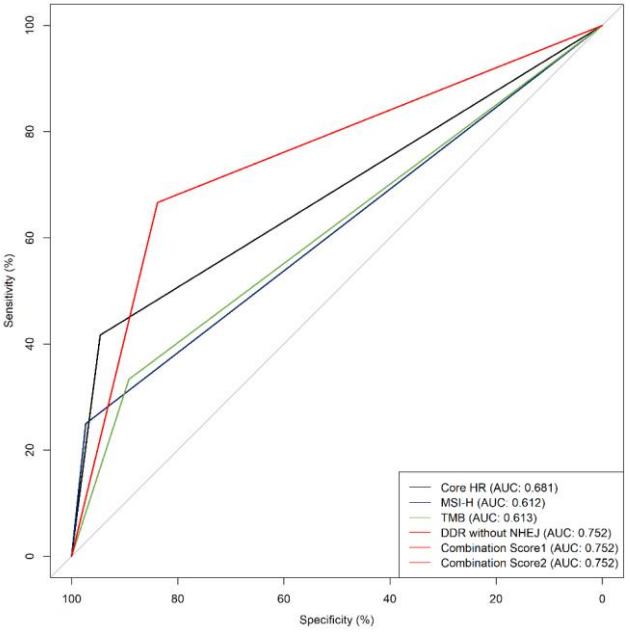


Figure 14. ROC curve and AUC in each factor. AUC : Area under the ROC curve.

As a result of analyzing the importance and olaparib sensitivity predictive ability of each factor, core HR alteration and DDR alteration excluding NHEJ were similarly important (2.53 vs 2.48). (Table 4) Based on the weight, a score was created by combining DDR alteration excluding NHEJ and genomic instability markers. However, in the case of TMB, since the weight is low, both scores with and without TMB were analyzed. The combined scores were calculated by adding the weights corresponding to each factor. The cutoff was set to 2 points using the ROC curve and Youden index. The sensitivity and specificity of the two scores were the same, and it was confirmed that TMB did not affect the score. The combined score was all the same as DDR alteration except for NHEJ which had the highest AUC. (Table 5, figure 14) It was confirmed that DDR alteration excluding NHEJ could sufficiently predict olaparib efficacy.

IV. DISCUSSION

Here we show that DDR related gene alteration pattern and status of genomic instability markers in gastric cancer cell lines, and that combined scoring was performed using DDR alteration, TMB, and MSI status for olaparib sensitivity prediction.

Drug development is also important, but it is also important to predict the effect and select the patient group that will benefit. Particularly, PARP inhibitor is a blatant drug and was developed for patients with a germline *BRCA1/2* mutation. Olaparib is a first-in-class PARP inhibitor approved for patients with advanced ovarian, breast and pancreatic cancer, particularly those with a germline *BRCA1/2* mutation.[24] Nevertheless, it is effective in patients who don't have any *BRCA1/2* mutations, and also there are many cases in which the olaparib does not make benefit even though patients have a *BRCA1/2* mutation. However, core HR gene mutation is still focused on selecting patients that will benefit from olaparib. Therefore, research is needed to profile DDR related genes with a wider range than core HR for predicting olaparib efficacy.

It is widely known that cancers with mutations in DDR have genomic instability. Recently, research results have been published that DDR mutation cancer has a high TMB which is a genomic instability marker. Nevertheless, very few studies have analyzed the relationship between genomic instability markers and olaparib sensitivity. Although there is a clinical study that there is no difference in olaparib response between MSI-H and MMS groups, DDR alteration was not considered at all.[25] Therefore, in this study, not only the individual predictive abilities of DDR gene alteration and genomic instability markers but also the combined scores according to their importance were confirmed. In addition, since the genomic instability marker is a predictive biomarker for immunotherapy, profiling will be important evidence for suggesting a combination therapy of olaparib and immunotherapy in gastric cancer.[26,27]

In forty-nine GC cell lines, the cell lines with DDR alteration were 40.81% (20/49). Ten of 13 (76.92%) HR altered cell lines and all of BER/NER altered

cell lines and MMR altered cell lines have alterations in different DDR subpathways. On the other hand, only five out of 11 NEHJ altered cells (45.45%) have alterations in other DDR subpathways. Similarly, unlike other DDR subpathways, the NHEJ altered group did not show significant differences in olaparib sensitivity and TMB compared to the wild type group. This seems to be because a choice between NHEJ and HR occurs in repairing DSB, so NHEJ deficiency cell lines repair DSB dependently on HR without DNA error.[28]

In this study, the DDR altered cell lines except for NHEJ were more sensitive to olaparib compared to the wild type group, and TMB was also high. In addition, TCGA subtypes, which are characterized by genomic instability, EBV and MSI, were more sensitive to olaparib compared to other subtypes. Contrary to expectations, even considering the genomic instability marker, the predictive ability of olaparib efficacy did not improve. In the case of MSI-H, all four cell lines have DDR alteration, so it is not expected to affect the sensitivity prediction. In the case of TMB, the weight is low, so it is not expected to significantly affect the combined score compared to DDR alteration. Nevertheless, compared to core HR, DDR alteration excluding NHEJ has higher sensitivity, larger AUC, and more than twice the number of cell lines. Therefore, it is necessary to check the DDR alteration by extending the gene rather than the core HR to predict olaparib sensitivity.

This study has several limitations. First, there are cell lines that are sensitive to olaparib even in the absence of DDR alteration and cell lines that are resistant to olaparib despite having an alteration in DDR. (Fig 12F) Also, since all MSI-H cell lines have alterations in DDR, it is not clear how much MSI status affect the prediction of olaparib sensitivity. Therefore, a larger-scale study in gastric cancer is needed.

V. CONCLUSION

We provide information on DDR alteration including gene mutation and methylation status and genomic instability markers for *in vitro* studies of PARP inhibitors, DNA damaging agents, and cancer immunotherapy. In this study, we suggest that DDR alteration should be identified beyond core HR to predict olaparib efficacy in GC.

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ABSTRACT(IN KOREAN)

위암에서 olaparib 감수성 예측 인자로써 DNA 손상 복구 유전자 변이와 현미부수체 불안정성 및 종양변이부담에 대한 평가

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황 지 현

암의 많은 원인 중 하나인 DNA 손상 복구 경로의 기능 장애이다. 돌연변이, 메틸화 또는 기타 이유로 인해 상동성 재조합 기능에 결핍이 있는 암은 PARP 억제제의 감수성과 유전체 불안정성이 증가하는 것으로 알려져 있다. 또한, DNA 손상 복구 경로에 결핍이 있는 암 역시 비슷한 특징을 갖는 것으로 보고되고 있다. 게다가, 일부 연구에서는 현미부수체 불안정성이 높거나 종양 변이부담이 높은 암과 같이 돌연변이가 많은 암의 경우 DNA 손상 복구 경로 기능에 결핍이 있을 확률이 높다. 그럼에도 불구하고 많은 연구가 여전히 olaparib 감수성 예측 인자로서 주요 상동성 재조합 기능 관련 유전자 돌연변이에만 초점을 맞추고 있다. 본 연구는 주요 상동성 재조합 기능 관련 유전자뿐만 아니라 DNA 손상 복구 경로 관련 유전자 돌연변이 및 유전체 불안정성 예측 인자와 49개 위암 세포주에서 olaparib 감수성과의 연관성을 확인한다.

Olaparib의 효능을 확인하기 위해 세포주를 olaparib으로 5일간 처리한 후 CCK-8 분석을 수행하였다. IC_{50}은 CalcuSyn 소프트웨어를 통해 계산되었으며, 세포주는 IC_{50}이 10μM

미만이며 10μM에서 억제율이 50% 이상일 때 olaparib에 민감하다고 분류된다. 그런 다음, 표적 시퀀싱을 이용하여 49개의 DNA 손상 복구 경로 관련 유전자, 현미부수체 불안정성 상태 및 종양 변이부담을 확인했다. *BRCA1, RAD51C* 및 *MLH1* 메틸화는 bisulfite 시퀀싱에 의해 확인했다.

그 결과, 20개의 세포주에서 16개의 DNA 손상 복구 경로 관련 유전자에서 돌연변이가 있음을 확인했다. 전체 세포주 중 12개의 세포주는 olaparib에 감수성이 높았고, DNA 손상 복구 경로 유전자의 변이군은 olaparib에 더 민감했다 (p = 0.034). 구체적으로 상동성 재조합 기능과 DNA 불일치 복구 기능에 변이가 있는 세포주는 야생군에 비해 olaparib에 민감했다 (p = 0.005, p = 0.018). 흥미롭게도 비상동말단연결 기능 유전자에 변이는 olaparib 민감성에 큰 영향을 미치지 않았다. 종양 변이부담은 세포주 (범위 = 1.45 ~ 61.03, 중앙값 = 8.70)에 분포되어 있다. 흥미롭게도, olaparib 감수성 그룹은 저항성 그룹보다 TMB가 더 높았다 (중앙값 = 15.3 대 10.9, p <0.0001). 4개의 세포주 (SNU-1, SNU-638, IM95m, NUGC-3)는 현미부수체 불안정성을 보였으며 모두 MLH1 단백질 발현이 없었다. 현미부수체 불안정성 세포주는 다른 세포주보다 상당히 높은 종양 변이부담을 가졌다 (중앙값 : 47.96 대 8.72, p <0.0001). 3개의 현미부수체 불안정성 세포주는 olaparib에 민감하였으나 현미부수체 불안정성 세포주 중 종양 변이부담이 가장 낮은 NUGC-3 (29.07)은 내성이 있었다. 각 요인의 중요성과 올라파립 감수성 예측 능력을 분석 한 결과, 주요 상동성 재조합 기능 및 비상동말단연결을 제외한 DNA 손상 복구 경로 변이가

비슷하게 중요했으며 (2.53 대 2.48), 비상동말단연결을 제외한 DNA 손상 복구 경로 변이가 olaparib 민감성을 가장 정확히 예측했다.

우리 연구는 olaparib 민감성을 예측하기 위해 주요 상동성 재조합 기능변이뿐만 아니라 나아가 DNA 손상 복구 경로 관연 유전자 변이 역시 확인해야한다고 제안한다.

핵심되는 말 : PARP inhibitor, homologous recombination deficiency, DNA damage repair deficiency, genomic instability marker, gastric cancer